

Hyperhydricity reversal and clonal propagation of four-wing saltbush (*Atriplex canescens*, Chenopodiaceae) cultivated *in vitro*

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Abstract. *In vitro* propagated shoots of four-wing saltbush (*Atriplex canescens*, Pursh Nutt) showed severe symptoms of hyperhydricity. We show that the reversion of hyperhydric *A. canescens* shoots to normal shoots was significantly affected by the presence of inorganic nitrogen in the culture vessel. When the culture vessel was vented or when ammonium nitrate was deleted from Murashige and Skoog basal medium, rates of reversion were significantly higher. Although statistically significant differences were evident when comparing vented v. non-vented treatments for each medium, the modified culture medium with vented closures was consistently the best treatment, showing a total cumulative frequency of 39.7% reversion to normal morphology, compared with a total cumulative frequency of 7.1% observed in the control treatment. Resulting normal shoots also showed significant improvements in further manipulations, including rooting *in vitro*, transplantation to soil and survival in native sites.

Introduction

Atriplex is a cosmopolitan genus with a high number of species distributed in all arid and semi-arid zones of the world. *Atriplex canescens* has been used extensively as a forage shrub in marginal agricultural lands of South Africa, Australia, the Middle East and North America. (Colomer and Passera 1990). *Atriplex* species supply high protein browse for livestock, with the content in *A. canescens* and related species ranging from 16.7 to 25% crude protein, on a dry matter basis (Khalil *et al.* 1986; Colomer and Passera 1990). Tissue culture technology is a powerful technique for clonal mass propagation of elite genotypes of four-wing saltbush for revegetation and phytoremediation programs in western arid rangelands in the United States of America (USA) (Salo *et al.* 1999; Bean *et al.* 2004; Sanderson and McArthur 2004). These regenerated clonal propagates can also be used as a source of genetically uniform biological material for basic research on *Atriplex* biology.

Hyperhydricity, or vitrification, is an induced physiological disorder and common problem of *in vitro* cultured plants (Debergh *et al.* 1992). Leaves and stems of hyperhydric plants are thick, distorted and brittle with a glassy (vitrified) appearance. Hyperhydricity is described as a nitrogen detoxification process in which carbohydrates are diverted from cellulose and lignin synthesis to amino acid biosynthesis, resulting in severe anatomical and biochemical abnormalities (Kevers *et al.* 1984; George 1996). This condition disrupts normal plant anatomy and physiology, and prevents rooting and establishment of *in vitro* propagated plants. Hyperhydric plants also exhibit non-functional stomata and an incompletely formed epidermis (Ziv *et al.* 1987; Ziv 1991;

Schloupf *et al.* 1995). Hyperhydric tissue also suffers from bioenergetic arrest (Franck *et al.* 2001).

Causal explanations for the occurrence of hyperhydric conditions include high humidity and supraoptimal quantities of inorganic and organic supplements (George 1996; Hazarika 2006). For example, media rich in NH_4^+ ions induce hyperhydricity in willow. (Daguin and Letouse 1984; Daguin and Letouse 1986; Pâques 1991). However, supplementation of tissue culture medium with fish protein hydrolysates, a reduced form of nitrogen, prevented hyperhydricity and improved chlorophyll content in oregano shoot cultures (Andarwulan and Kalidas 1999; Eguchi *et al.* 1999). The most important environmental factor for the induction of hyperhydricity appears to be the continuous culture of plants in high relative humidity (Kidelman Dantas de Oliveira *et al.* 1997). Ventilation or aeration reduces relative humidity in the culture vessel and dilutes evolved gases, such as ethylene (Kevers and Gaspar 1985; Fal *et al.* 1999; Saher *et al.* 2004; Lai *et al.* 2005), which might contribute to the induction of hyperhydricity.

Hyperhydricity has been reported during *in vitro* propagation of several species of *Atriplex*, and cultured shoots may show a frequency of 30–90% (Wurtele *et al.* 1987; Tripathy and Goodin 1990; Kenny and Caligari 1996). Wochok and Sluis (1980) clearly describe the occurrence of hyperhydricity in shoot cultures of *Atriplex*, but fail to identify the phenomenon. Even though hyperhydricity is a frequently observed phenomenon in *Atriplex* propagation protocols, this issue has been poorly addressed. One of the most relevant problems of hyperhydricity is that only normal shoots were able to root.

Previous researchers have reported shoot proliferation from *Atriplex* explants, but root formation data were either omitted (Tripathy and Goodin 1990) or shown to occur at low frequencies (Wochok and Sluis 1980; Wurtele *et al.* 1987; Kenny and Caligari 1996). Hyperhydricity and ventilation, or hyperhydricity and ammonium have been reported in other plants (Daguin and Letouse 1984; Daguin and Letouse 1986; Fal *et al.* 1999). The aim of this research is to address hyperhydricity in *A. canescens* by modifying multiple key culture factors, including salt composition, supplemental additives and use of ventilated culture vessels. We also report *Atriplex* rooting efficiency with nodal or internodal cuts of the reverted shoots.

Materials and methods

Culture initiation

Four-wing saltbush seeds were surface sterilised with 95% ethanol for 1 min, 2.6% sodium hypochlorite for 7 min, then rinsed three times in sterile distilled water. Seeds were placed on hormone-free, low ionic concentration medium, based on White (1934). The *A. canescens* seedlings were then used to initiate shoot cultures by transferring them to shoot proliferation medium consisting of standard Murashige and Skoog (MS) basal salts (Murashige and Skoog 1962), with 11.42 μM indole-3-acetic acid (IAA), 18.58 μM 6-furfurylaminopurine (Kinetin), vitamin in the L2 formulation of Phillips and Collins (1979) and 30 g L^{-1} sucrose, solidified with 0.8% agar. Cultures were grown in 100 \times 25 mm polystyrene petri dishes and sealed with Parafilm to induce hyperhydricity. They were subcultured to fresh medium every 4 weeks. This medium represents the standard control medium.

Growth condition treatments

Hyperhydric *A. canescens* shoots were transferred to standard control shoot proliferation medium, or to modified MS medium (ammonium-free MS with NH_4NO_3 excluded from the original major salts composition) plus 4.40 g L^{-1} of casein hydrolysate to provide an equivalent amount of total nitrogen comparable to the standard MS formulation. All experimental treatments were solidified with 5.0 g L^{-1} of Agargel (Sigma Chemical Co., St Louis MO, USA). Growth regulator composition consisted of 24.61 μM 6-(γ - γ -dimethylallylamino) purine (2iP). The pH of the medium was adjusted to 5.8 ± 0.05 , before autoclaving. The autoclaved medium was then dispensed in polycarbonate Magenta GA7 vessels (Magenta Corp., Chicago, IL, USA). Culture boxes were closed either with standard Magenta GA7 vessel covers or vented lids with a 10 mm polypropylene membrane (0.22 μm pore size, Magenta). All cultures were incubated at $28 \pm 1^\circ\text{C}$ under continuous fluorescent light (14–18 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Hyperhydric *A. canescens* shoots, under propagation conditions, generate multiple bud clumps. These anatomically interconnected bundle shoots propagate and remain in a reduced morphological rosette-like condition with a completely suppressed apical dominance. Clusters of *Atriplex* rosettes exhibited classic hyperhydric characteristics, where leaves were abnormally thick, translucent and watery with a glass-like appearance. Clusters were used because individualised

rosettes failed to grow and eventually died. Nine hyperhydrated multiple bud formations were used per Magenta vessel, with six replications per treatment. Hyperhydric *A. canescens* shoots that had visually reverted to a normal appearance (reverted shoots) were removed from vessels at the end of each culture period (30 days per period). Reverted shoots were defined as all shoots that elongated, reassumed apical dominance and overcame the rosette habit of growth.

Statistical analysis

Cultures were statistically evaluated, then subcultured to fresh medium every 30 days. The independent variables tested included standard or modified MS and non-vented or vented lids. The frequency of hyperhydric shoots that reverted to normal shoots per treatment was the dependent variable. Analyses of variance (ANOVA) were calculated after each culture cycle (30 days) by using the cumulative frequencies for the reverted shoots from 30 to 150 days with Statistical Analysis System software (SAS). A Duncan's multiple range tests was carried out for each ANOVA test (SAS 1989a, 1989b, 1999).

Rooting of reverted shoots

Reverting shoots from all treatments were transferred to optimal conditions, as determined by experimentation, and passaged every 4 weeks for 3 months to establish them on the same background propagation conditions. Reverted single stems were then excised and transferred to low ionic strength medium, generally used for root initiation (White 1934; George 1996) containing 2.46 μM of indolebutyric acid (IBA), 30 g L^{-1} of sucrose and solidified with 2.5 g L^{-1} of Phytigel (Sigma Chemical Co., USA) to evaluate root initials. The pH was adjusted to 5.8 ± 0.05 before autoclaving. Sterilised medium was then dispensed in 107 L \times 107 W \times 96 H mm LifeGuard polycarbonate culture boxes closed with vented lids (22 mm diameter and a 0.3 μm pore size; Osmotek Ltd, Israel). Two different types of explants from the reverted shoots were used in this rooting experiment. The first type of explant for rooting included 25 shoots that were excised between two leaf nodes (internodal cutting), whereas the second type of explant included 25 shoots where the stem section was cut just below a node (nodal cutting). All shoots were at least three nodes long. Rooted shoots were transferred to hydrated 30 mm JiffyPots peat pellets. The hardening process was carried out placing the shoots in peat pellets, inside Magenta boxes capped with vented lids, with a 10 mm polypropylene membrane (0.22 μm pore size, Magenta). This acclimatisation strategy was compared with rooted shoots transplanted to germination flats covered with a clear plastic cover and commercial soil mix.

Results

Atriplex canescens shoots, on standard propagation MS medium (control medium), in sealed petri dishes, showed severe hyperhydricity symptoms in 100% of cultures. Hyperhydricity reversion did occur in the control medium in sealed petri dishes, but at a very low frequency (data not shown). After induction, hyperhydric *Atriplex* bundle shoots were transferred to Magenta boxes with standard and modified media and with non-vented or

vented lids. Successful reversion to visually normal shoots occurred with all Magenta vessel cultures.

Statistically significant differences were evident when comparing vented *v.* non-vented for each medium treatment for all culture cycles. The reversion frequencies observed in every culture cycle were consistently higher in both vented treatments from 60 to 150 day periods (Fig. 1). The modified culture medium with vented closures was consistently the best treatment with a total cumulative frequency of reverted shoots of 39.7% (73 reverted shoots of 184 total reverted shoots), compared with a total cumulative frequencies of reverted shoots of 7.1% (13 reverted shoots of 184 total reverted shoots) observed in the standard control medium, non-vented treatment (see 150 days in Fig. 1).

The use of vented lids as main factor had significant effects on the reversion rate. Vented closures consistently resulted in significantly higher frequencies of reverted shoots after the initial 30-day period to the 150-day period. There was an observed two-fold increase in cumulative frequencies of reverted shoots over 30–150 days for the vented effect (67%, 123 reverted shoots of 184 reverted shoots) when compared with the non-vented effect (33%, 61 reverted shoots of 184 reverted shoots) (Fig. 2).

The use of the modified medium also had significant effects on shoot reversion rates. With the removal of ammonium from the medium formulation, the modified medium treatment gave a two-fold increase in total reversion rates with 121 (66%) and 63 (34%) total reverted shoots of 184 respectively (Fig. 3).

The statistical interaction between medium treatment and venting, as main effects, was evaluated for every analysis of variance. In all cases this interaction was not significant (data not shown). This shows that there was no synergistic effect in the use of both modified medium formulation with vented lids, and that the medium treatment and venting have independent causative effects on hyperhydricity. Therefore, to overcome hyperhydricity in *Atriplex*, improvements in both media formulation and ventilation of vessels are needed to propagate functional *Atriplex* shoots.

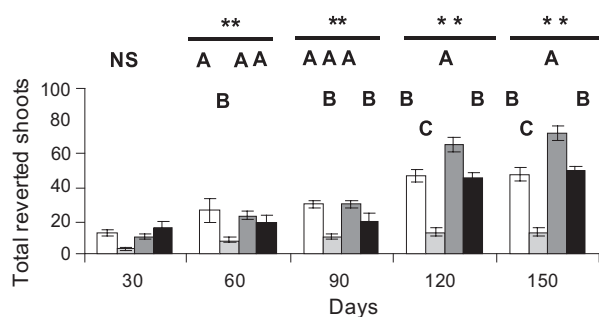


Fig. 1. Cumulative reversion of shoots per treatment of *Atriplex canescens* from 30 to 150 days. Cumulative reversions were calculated by adding together the total reverted shoots per treatment for each of the five culture cycles. Standard vented (open bars), standard non-vented (light grey bars), modified vented (dark grey bars), modified vented (black bars). (1) ANOVA significant at $P < 0.05$ (*) or $P < 0.01$ (**), NS = non-significant, (2) treatments with the same letter are not significantly different from each other by Duncan's multiple range tests.

The use of vented lids, together with modified medium, showed the highest potential to produce newly reverted shoots each culture cycle. These reverted shoots recovered their characteristic ashen-green colour and elongated internodes with enhanced mechanical rigidity. Subsequent culture of reverted shoots in modified medium formulation with vented lids produced multiple normal looking shoots with a similar appearance to *in vivo* grown plants. The mean number of shoots

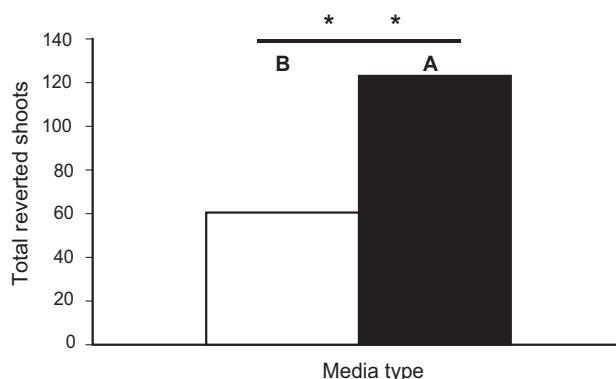


Fig. 2. Cumulative frequencies of reverted shoots of *Atriplex canescens* by media formulation from 30 to 150 days. Cumulative frequencies of reverted shoots were calculated by adding up the total reverted shoots per media type for each of the five culture cycles. Standard MS (open bars), modified MS (black bars). (1) ANOVA significant at $P < 0.05$ (*) or $P < 0.01$ (**), (2) treatments with the same letter are not significantly different from each other by Duncan's multiple range tests.

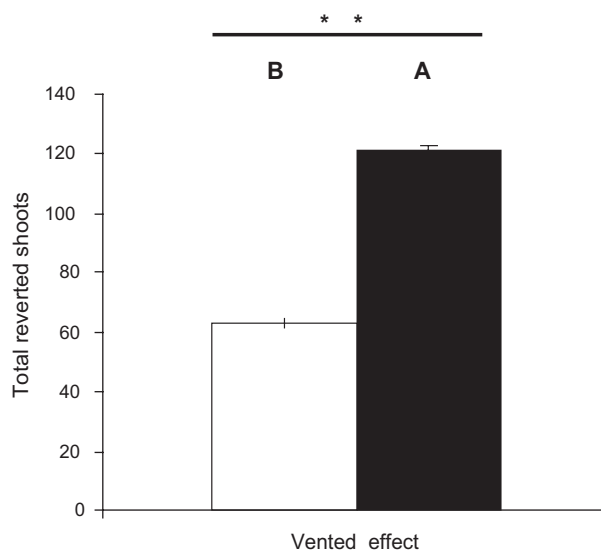


Fig. 3. Cumulative frequencies of reverted shoots of *Atriplex canescens* by vented effect from 30 to 150 days. Cumulative frequencies of reverted shoots were calculated by adding up the total reverted shoots per vented *v.* non-vented effect for each of the five culture cycles. Non-vented (open bars) and vented (black bars). (1) ANOVA significant at $P < 0.05$ (*) or $P < 0.01$ (**), (2) treatments with the same letter are not significantly different from each other by Duncan's multiple range tests.

per reverted explant per culture cycle was $3.5\times$, a value similar to that for rapid *in vitro* multiplication of *A. canescens* reported by Wurtele *et al.* (1987).

Reverted shoots from all treatments showed no difference in rooting frequencies (data not shown). Therefore, the explants were used to assess whether *Atriplex* develops roots at a higher frequency as nodal or internodal cuttings. The presence of a node at the base of cuttings may enhance rooting (Marks and Sally 2000), whereas the absence of a node can decrease the number of rooted shoots (Ferreira and Handro 1988). The highest rooting frequencies for reverted *Atriplex* shoots in this study were accomplished with the internodal cut. In all, 22 of 25 (88%) internodal cuttings rooted, whereas only 15 of the 25 nodal cuttings rooted (56%).

Propagation, rooting and acclimatisation of *Atriplex* shoots in vented culture vessels allowed acclimatisation with negligible stress during transplant to soil, as well as high survival rates. The strategy of acclimatisation of rooted shoots in peat pellets in vented Magenta boxes yielded higher transplant survival (82%) when compared with hardening of shoots in germination flats with clear plastic covers (65%). After growing during the winter in a greenhouse, established plants were transplanted to natural sites the following spring. Transplanted plants flowered that spring and were determined to be a male clone.

Discussion

Relative high humidity, the environmental factor most relevant to hyperhydricity induction, is inherent in sterile, sealed *in vitro* processes. Nitrogen rich media formulations also impose intense nitrogen assimilation processes with a high carbon skeleton demand on cultured tissues, diverting valuable resources from other key carbon metabolic pathways, such as cellulose and lignin formation (Daguin and Letouse 1984; Kevers and Gaspar 1985; George 1996), resulting in hyperhydricity. Our strategy to alleviate this metabolic disorder in *A. canescens* cultures consisted of substituting high NO_3^- and NH_4^+ ion concentration with casein hydrolysate, which provided a reduced non-toxic source of nitrogen, and by the use of vented lids to reduce relative humidity within the tissue culture vessel. In combination, vented lids and modified nitrogen formulation resulted in significantly higher reversion of *A. canescens* than either alone.

Reversion did occur in both non-vented and control medium formulations, although at a significantly lower frequency. This is potentially due to metabolism of high ammonium concentrations as cultures aged, dispersal of accumulated tissue culture gases during each 30 day transfer cycle and loss of water over time, which causes the salts, sugars and agar to become more concentrated, decreasing water potential in the medium and causing the relative humidity to fall (Gaspar 1991; George 1996). The reduction of the water potential in media culture by increasing agar concentrations has also been reported as a hyperhydricity cure (Pasqualetto *et al.* 1986; Brand 1993; George 1996).

The induction of hyperhydricity during tissue culture procedures is generally unwelcome and problematic. Gaining knowledge about the causes and remedies of this physiological disorder could yield practical applications. John (1986) induced

and reverted hyperhydricity deliberately in *Picea sitchensis* because he found that hyperhydric and reverted shoots produced a significantly higher number of shoots when compared with control. George (1996) and Kidelman Dantas de Oliveira *et al.* (1997) have subsequently found that hyperhydric plants behave like cytokinin-habituated cultures. Therefore, hyperhydricity can be manipulated to boost multiplication rates without the use of growth regulators. Another practical application derived from the manipulation of the hyperhydricity phenomenon has been reported by Zimmerman (1996). Hyperhydric meristems are shorter and wider, with fewer developing primordial leaves. All cells showed a generalised reduced lignification (hypolignification) with poor cell differentiation. These anatomical abnormalities make them more amenable for genetic transformation.

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